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Generation of Transgenic Mice with Overexpression of Mouse Resistin

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ABSTRACT

The hormone resistin is associated with type Ⅱ diabetes mellitus in rodent model. Resistin impairs glucose tolerance and insulin action. A new class of anti-diabetic drugs were called thiazolidinediones (TZDs) downreguates a resistin. Resistin gene expression is induced during adipocyte differentiation and resistin polypeptide is secreted by adipocytes. But, the correlation between increased adiposity and resistin remains unknown. The objectives of this study was to clone a mouse resistin cDNA and to generate transgenic mice overexpressing mouse resistin gene. The pCMV-mus/resistin gene was prepared from previous recombinant pTargeT^{TM}-mus/resistin by digestion of Bgl Ⅱ, and has used for microinjection into pronuclei of one cell embryos. Mouse resistin expression was detected in transgenic F_1 mice by RT-PCR. The transgenic mouse with resistin gene expression has heavier body weight which was measured higher level of plasma glucose than that of normal mouse. And in diet-induced experiments, in fasting group, resistin expression was higher than that of re-feeding group.

This result demonstrates that the resistin gene overexpressing mice may be became to obesity and be useful as an animal disease model to be diabetes caused by insulin resistance of resistin.

(Key words : Resistin, Obesity, Diet-induced, Transgenic)

Ⅰ. INTRODUCTION

Adipose tissue is the largest reservation of fuel in the body, storing energy in the form of triacylglycerides. The switch from energy storage to mobilization within adipocytes is regulated by hormonal signals from other tissues and organ, including the pancreas (insulin), the sympathetic nervous system (catecholamines) and the adrenal glands (glucocorticoids). These adipocyte-drived hormone, the elevated levels of which in obestiy are probably causally related to reduced insulin action in peripheral tissues (Boden et al., 1997).

One of these adipocyte-drived hormones, resistin is related with insulin resistance that is associated with type Ⅱ diabetes mellitus. Type Ⅱ diabetes mellitus is characterized by target-tissue resistinace to insulin (Taylor et al., 1999) that cannot be overcome by β-cell hypersecretion and is strongly associated with insulin resistance. However, the correlation between increased adiposity and insulin resistance remains unknown (Kahn et al., 1996).

Resistin expression is induced during adipocyte differentiation, and the resistin polypeptide is specifically expressed and secreted by adipocytes. TZD

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treatment downregulates an adipocyte gene that contributes to insulin resistance. Resistin mRNA is induced markedly during adipocyte differentiation of 3T3-L1 cells (Claire et al., 2001). Resistin is expressed almost exclusively in white adipose tisse (Claire et al., 2001; Kim et al. 2001; Holocomb et al. 2001). Resistin mRNA levels varied as a function of white adipose depot and gender, with the highest level of expression in female gonadal fat (Claire et al., 2001).

Although, there are many research about a new adipocyte-specific secreted hormone, resistin, but we are still unknown about it's receptor, downstream signaling pathway, molecular target, and so on. Moreover, there are no any reports about generation of overexpressing resistin gene in mice as an animal disease model system.

Therefore, this study was performed to clone the 555 bp of mouse resistin and to generate transgenic mice carrying the cloned mouse resistin gene. These results is formed by studies performed in mice that may help to study in function of human resistin and difference of mouse and human resistin on the mechanism of obesity-related insulin resistance in these two species.

Ⅱ. MATERIALS AND METHODS

1. Expression of Mouse Resistin in Diet-induced Mice

5-weeks old female B6D2F1 mice (Daehanbiolink Co., Korea) were housed conventionally in animal room with constant temperature (22～24℃), humidity (50～60%) and with a 14/10-h light/dark cycle (light an at 9:00 a.m.). Then mice were divided two treatment groups (1. fasting, 2. refeeding, n=3 per group). 'S'group mice were sacrified following for 24 hr fast, and their abdominal fat pads were taken. For 24 hr fasting, then 'R' group mice were refed a regular mice chow for 24 hr. After this dietary manipulation, their abdominal fat pads were taken. Plasma glucose was measured by One Touch Basic Complete Diabetes Monitoring System (Lifescan Inc., USA). The relative levels of mRNA of each treated mouse were assessed by 1% agarose gel electrophoresis following RT-PCR. Total RNA was extracted from adipocytes of each mouse by using Trizol (Gibco, USA). Isolated total the first strand cDNA was synthesized by using AMV Reverse Transcriptase First-strand cDNA Synthesis kit (Life Sciences, USA). For PCR reaction, primer sequences are as follow: forward primer 5'-TCAACAAGAAGGAGCTGTGG-3'; reverse primer 5'-GTATGTGTGCTTGTGTGTGG-3'; product size, 555 bp. The mixture of the PCR was as follow: 5 μl of $\times$ 10 *Ex Taq* polymerase, 100 pM of sense and antisense primer, and 2 μl of RT reaction mixture. Thermal cycling profiles for amplifying each cDNA were as follow: denaturation at 94℃ for 30 sec, annealing at 55℃ for 30 sec, and extention at 72℃ for 30 sec for 30 cycles. The PCR products were subjected to 1.0% agarose gel, and the quantification of electrophoretic bands were featured by BIO-1D Image Analysis system. Statistical analysis was performed using an equal variance Student's t-test with $P<0.05$ considered significant by using SAS software.

2. Transgenic Analysis

1) Cloning of the Mouse Resistin Gene

Total RNA was extracted from mouse adipose tissues by using Trizol (Gibco, USA). Total isolated RNA was reversely transcribed to synthesize the first strand cDNA by using AMV Reverse Transcriptase First-strand cDNA Synthesis kit (Life Sciences, USA). Then, to clone mouse resistin gene, PCR amplication was performed by using primer for mouse resistin (5'-TCAACAAGAAGGAGCTGTGG-3'and 5'-GTATGTGTGCTTGTGTGTGG-3').

These primers were designed to according to the published mouse mRNA sequence available from Genbank sequence (accession no. AF323080) and synthesized in Bioneer Co., Korea. The mixture of the PCR was as follow: 5 μl of ×10 *Ex Taq* buffer, 4 μl of dNTP mixture (2.5 mM each), 2.5 U of TaKaRa Ex Taq polymerase, 100 pM of sense and antisense primer, and 4 μl of RT reaction mixture. Thermal cycling profiles for amplifying cDNA was as follow: denaturation at 94℃ for 30 sec, annealing at 55℃ for 30 sec, and extention at 72℃ for 30 sec for 30 cycles. To confirm exact sequence of the cloned gene, the purified cDNAs of mouse resistin was subcloned into the T overhang site of pCR®2.1-TOPO vectors (Invitrogen, USA). DNA sequencing was performed by Macrogen, Co., Korea.

2) Construction of DNA Expression Vector

The single deoxyadenosine (A) to the 3' ends of PCR product ligated with pCR®2.1-TOPO vector that has single, overhanging 3'deoxythymidine (T) residues. Then, the recombinant TOPO-mus/Resistin was digested with BamH Ⅰ and Not Ⅰ. For expression of the cloned mouse resistin cDNA, the digested musResistin ligated with the pTargeT™ vector (Promega, USA) digested with BamH Ⅰ and Not Ⅰ. The mammalian expression vector, pTargeT™ which contains the human cytomegalovirus (CMV) immediate-early enhancer/promoter region to promote, chimeric intron and SV40 polyadenylation signals. The ligation mixture was as follow: 1 μl of T4 DNA ligase ×10 buffer, 50 ng of pTarget vector, 50 ng of digested musResistin gene, 1 μl of T4 DNA ligase (TaKaRa). The ligation reaction was performed on a 16℃ water bath and incubated overnight. Then, the recombinant pTarget-mus/Resistin transformed into Novablue (DE3) competent cells (Novagen, Inc. USA) by heat shock method. After white colony was harvested from the Blue/White screening, the recombinant pTargtT-mus/Resistin was isolated by plasmid DNA purification system. The recombinant pTarget-mus/Resistin was digested with Bgl Ⅱ for the microinjection, and the digested 6.3 kb fragment cleaned up from 1.0% agarose gel by QIAquik® gel extraction kit (QIAGEN Inc., USA). The pTargeT-mus/Resistin vector was introduced into a total 2×10^5 Hela cells on 35 mm tissue culture plate by cationic lipid reagent -mediated transfection, Lipofectin® Reagent (Invitrogen). EGFP expression was examined under fluorescent microscope from pEGFP-N1 vector transfected Hela cells. The recombinant mouse resistin gene integration was examined by genomic DNA from the recombinant pTarget-mus/Resistin vector trnasfected Hela cells by PCR analysis.

3) Generation of Transgenic Mice

Mice of B6D2F1 (C57BL/6J × DBA/2J) F1 and ICR strains were purchased from Daehanbiolink Co., Korea. Female mice superovulated by an intraperitoneal injection of 5 IU pregnant mare serum gonadotropin and followed an intra-peritoneal injection of human chorionic gonadotropin 48 hr later. Mice were killed between 18 and 20 hr after hCG injection by cervical dislocation. The pronuclei of fertilized eggs were collected and the cumulus cells were removed in CZB medium containing 300 μg/ml of hyaluronidase (Sigma, USA). The one -cell eggs were centrifuged at 12000 rpm for 10 min at 37℃ to reveal the normally obscure pronuclei. Approximately 1 to 2 pl containing about 10 μg/ml of DNA solution was injected into the male pronucleus of the one-cell embryos. Microinjection was performed at ×400 with a Hoffman Modulation contrast Nikon objective on a Nikon inverted microscope (Eclipse TE 300; Nikon Inst., Japan) fitted with micromanipulator (Narishige Co., Japan). Survived embryos were incubated in CZB medium overnight in a 5% CO_2 incubator at 37℃

for growth to the two-cell stage. Then, the live eggs (1～2 cell stage) were transferred to the oviduct of pseudopregnant ICR mice by methods of Hogan et al (1986).

4) Analysis of Transgenes in Genomic DNA

From one week old F1 offsprings, tail biopsy was performed by clipping 0.5cm from each pup at the time of weaning. Each genomic DNA was extracted from the tail samples by phenol extraction methods (Hogan et al., 1986). Then PCR was performed by using primers (downstream: 5'-AGTGTATCATATGCCAAGTCCGCC-3' and upstream: 5'-CTCAAGACTGCTGTGCCT-3'). Primers were designed to detect sequence containing both a part of human cytomegalrovirus (CMV) promoter and resistin cDNA sequence. Thermal cycling profiles were as follows: denaturation at 94℃ for 30 sec, annealing at 70℃ for 1 min, and extension step at 72℃ for 30 sec for 40 cycles.

Ⅲ. RESULTS

1. Effects of Food Restriction in Resistin Gene Expression

Each of mice was sacrificed as after a fasting and a re-feeding after fasting. For studying the effect of food restriction on the expression of adipocyte resistin gene, six 20～22 g B6D2F1 female mice were sacrificed that were also fed on regular purina mouse chow. Total RNA was extracted from the isolated abdominal adipocytes of each mouse, using Trizol (Gibco, USA). The relative levels of mRNA of resistin and β-actin were assessed by RT-PCR. For the quantitative gene expression analysis, house keeping gene, β-actin was amplified simultaneously under conditions to resistin PCR. The mice after a 24 hr fasting and a re-feeding after fasting, showed the change in the quantities of their amplfied resistin. R group's resistin gene expression, as normalized with β-actin gene expression, was significantly lower than that of the F group. This result was showed that serum resistin levels were induced in mice with dietary manipulation.

2. Cloning of Mouse Resistin Gene

Total RNA was isolated from mouse adipocyte tissues and the 555 bp of mouse resistin cDNA was amplified by RT-PCR (Fig. 1). In order to produce reversely orientated mouse resistin cDNA, they were inserted into pCR®2.1-TOPO vectors. To confirm the exact sequence of cloned mouse resisitn cDNA, the PCR product was sequenced in Macrogen, Co., Korea. As a result of sequencing, the sequences of mouse resistin cDNA was correct (Fig. 2).

3. Construction of Mammalian Expression Vector

To induce the expression of the mouse resistin mRNA, they were inserted into pTargeT™ with

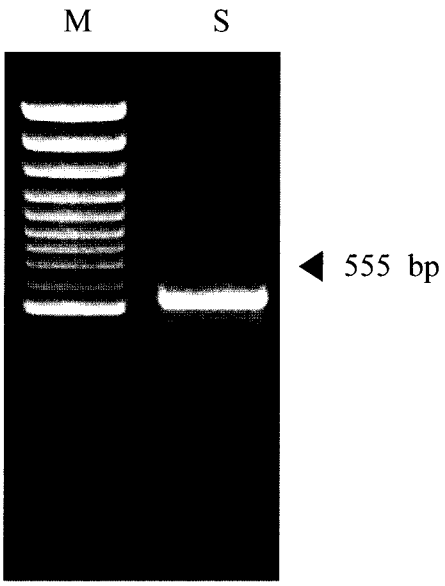

Fig. 1. PCR amplificaton of mouse resistin cDNA from adipose tissue. Lane M : 100 bp size marker, lane S : The 555 bp of PCR product.

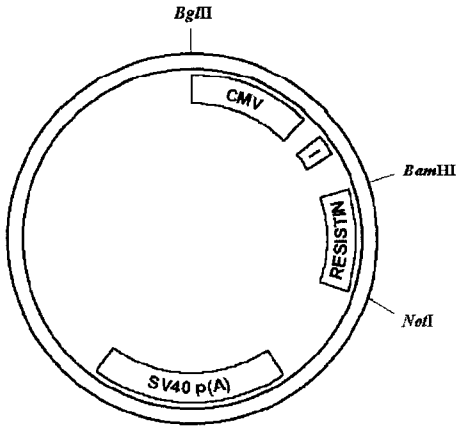

Fig. 2. Construction of pTargeT™ mouse resistin expression vetor. For microinjection, the expression vector was digested with *Bgl* II.

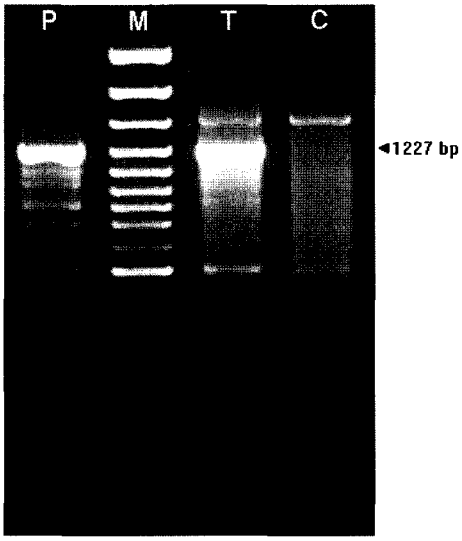

Fig. 3. Transfection of recombinant pTargeT™ /mouse resistingene on Hela cell was detected by PCR analysis. P : positive control, M : 100 bp size marker, T : transfected Hela cell, C: non-transfected Hela cell.

human cytomegalrovirus (CMV) promoter which also has chimeric intron and SV40 poly (A) (Fig. 3). The recombinant mouse resistin gene was

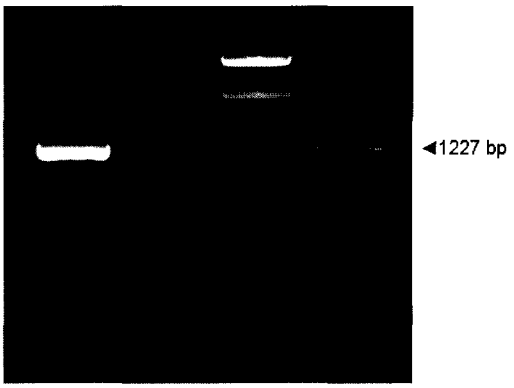

Fig. 4. PCR analysis of transgene for resistin gene. P: negative genomic DNA mixed with microinjected gene. M: 100 bp size marker. N: non-transgenic mice. S: transgenic mice with recombinant resistin gene.

confirmed by RT-PCR that expression of resistin mRNA were detected on Hela cells transfected with pTargeT-mus/Resistin vector, but not detected on non-transfected Hela cells (Fig. 4). Microinjection into pronucleus, the recombinant vectors were digested with *Bgl II*.

4. Screening of Transgenic Mice

The recombinant pTargeT-mus/Reistin was microinjected into pronuclei of total 1120 fertilized mouse zygotes and among them, 644 embryos were

Table 1. Production of transgenic mouse with mouse resistin gene overexpression by PN microinjection

No. of injected zygotes	No. of transferred embryos (%)	No. of offsprings (%)	No. of positive (%)
1120	644 (57.51)[a]	23 (2.05)[b]	1 (0.089)[c]

[a]: No. of transferred embryos/No. of injected embryos ×100.

[b]: No. of offsprings/No. of injected embryos × 100.

[c]: No. of positives/No. of injected embryos × 100.

Fig. 5. A transgenic mouse made by injecting recombinant mouse resistin gene into the pronucleus of the one-cell embryo of B6D2F1 hybrid. A; transgnic mouse, B; non-transgenic mouse.

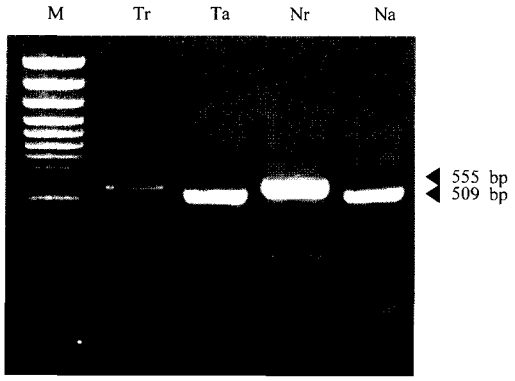

Fig. 6. Expression of mRNA in adipose tissue from transgeic F_1 mice by RT-PCR. Tr; 555 bp of resistin mRNA from transgenic F_1 mice Ta; 509 bp of β-actin mRNA from transgenic F_1 mice Nr; 555 bp of resistin mRNA from normal mice Na; 509 bp of β-actin mRNA from normal mice.

developed to the two-cell stage in the next morning (Table 1). These survived two-cell embryos were transfered to the oviduct of pseudopregnant foster mother. After 19~20 days, 28 live pups were delivered. At one week later, genomic DNAs were isolated from these pups and PCR was performed

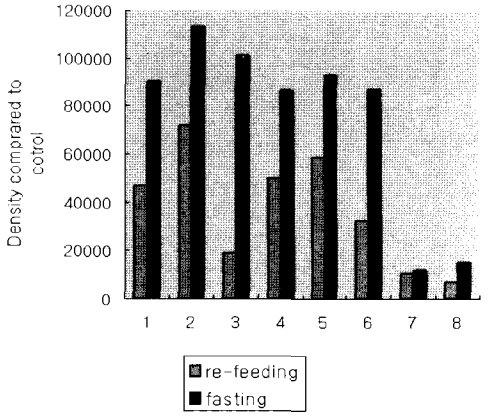

Fig. 7. Measurement of resistin gene expression *magnitude in mouse adipocytes. Amounts* of the total RNA of mouse adipocytes were used as template for RT-PCR with primer pairs specific for resistin and β -actin. Resistin gene expression levels of fasting group and re-feeding group.

with specific primers. As a result of PCR, one of transgenic mice with recombinant mus/Resistin were generated (Fig. 3).

Ⅳ. DISCUSSION

Resistin is secreted by rodent fat cells and was postulated to be an important relationship between obesity and insulin resistance, and resistin circulates in mouse serum, and it's level is increased markedly in both genetic and diet-induced obesity (Claire et al., 2001). For this reason, this study was investigated resistin hormone in transgenic mouse with mouse resistin overexpression.

This transgenic mouse was born with three other pups from one ICR surrogate mother that has higher body weight and glucose tolerance than same litter pups, and has an amount of fat pad in the abdomen. But it's resistin expression levels were lower than those of non transgenic mice.

Fasting group mice had higher resistin expression

levels than those of non-fasting group mice but glucose tolerance and body weight were lower in fasting group mice than those of non-fasting group.

The importance of these finding is examplified by several studies demonstrating that the levels of resistin expression correlates body weight (obesity) and insulin resistance as follow researches. Steppan et al. reported that resistin circulates in the mouse, with increased levels in obesity, and has effects on glucose homeostasis that oppose those of insulin.

A issue of great interest in this study is that resistin mRNA expression of adipose tissue was detected a small quantity in resistin gene overexpression mouse in contrast to non-transgenic mouse and also fasting group mice have higher resistin expression levels than those of non-fasting group mice. This result might be supported by Way et al. in obesity was related with decreasing resistin expression, but experiment of diet-induce mice was clashed with Way's opinions. Adipose tissue, are serve of energy, has played an essential role in mammalian evolution. Adipose tissue differs from other tissues in that its mass has considerable capacity to expand, which while beneficial in decreasing the risk of starvation, increases the risk of predation (Vernon et al., Domest Anim Endocrinol., 2001). So that mean, at this transgenic mouse, unlike normal mice if it has large-sized adipose tissue that has the same amount resistin expression levels, an equal amount isolated fat pads have small number of fat cells. The other words, resistin expression levels were induced an amount of adipocyte differentiation level. Such transgenic mice and diet-induced mice experiments was supported in previous investigation of the results.

Finally, more considerations are needed to compare to the protein level of the resistin in transgenic mice, situation of germline transmission, and physiological effects of the overexpressed resistin and other obese genes on the mice.

Ⅴ. REFERENCES

1. Auwerx, J. et al. 1996. Transcription, adipocyte differentiation, and obesity. J. Mol. Med., 74: 347-352.
2. Banerjee, R. R. et al. 2001. Dimerization of resistin and resistin-like molecules is determined by a single cysteine. J. Biol. Chem. M103109200.
3. Blagoev, B. et al. 2002. Inhibition of adipocyte differentiation by resistin like molecule α(RELM α): Biochemical characterization of its oligomeric nature. J. Biol. Chem.
4. Claire, M. S. et al. 2002. Resistin and obesity -associated insulin resistance. Trends Endocrinol. Metab. 13:18-23.
5. Claire, M. S. 2001. A family of tissue-specific resistin-like molecules. PNAS 98:502-506.
6. Engert, J. C. et al. 2002. 5′ flanking variants of resistin are associated with obesity. Diabetes, 51:1629-1634.
7. Fasshaurer, M. et al. 2001. Tumor necrosis factor α is negative regulator of resistin gene expression and secretion in 3T3-L1 adipocytes. Biochem. Biophys. Res. Commun., 288:1027-1031.
8. Hartman, B. H. et al. 2002. Mechanisms regulating adipocyte expression of resistin. J. Bio. Chem. M201451200.
9. Holocomb, I. N. et al. 2001. FIZZ1, a novel cystein-rich secreted protein associated with pulmonary inflammation, defines a new gene family. EMBO J., 19:4046-4055.
10. Hotamisligil, G. et. al. 1993. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science, 259:87-91.
11. Juan, C. C. et al. 2001. Suppressed gene expression of adipocyte resistin in an insulin -resistant rat model probably by elevated free

fatty acids. Biochem. Biophys. Res. Commun., 289:1328-1333.
12. Kahn, B. B. et al. 2000. Obesity and insulin resistance. J. Clin. Invest., 106:467-472.
13. Kahn, C. R. et al. 1996. A Genetics of non -insulin-dependent type 2 diabetes mellitus. Annu. Rev. Med., 47:509-531.
14. Kim, K. H. et al. 2001. A cysteine-rich adipose tissue-specific secretory factor inhibits adipocyte differentiation. J. Biol. Chem., 276:11252-11256.
15. Lay, S. L. et al. 2001 Decreased resistin expression in mice with different sensitivities to a high-fat diet. Biochem. Biophys. Res. Commun., 289:564-567.
16. Ling, C. et al. 2001. Increased resistin expression in the adipose tissue of male prolactin transgenic mice and male mice with elevated androgen levels. FEBS Letters, 507:147-150.
17. McTernan, C. L. et al. 2002. Resistin, central obesity, and type 2 diabetes. Reserch Letters, 359:46-47.
18. Morash, B. A. et al. 2002. Resistin expression and regulation in mouse pituitary. REBS Letters, 526:26-30.
19. Osawa, H. et al. 2002. Systematic search for single nucleotide polymorphisms in the resistin gene. Diabetes, 51:863-866.
20. Shao, D. et al. 1997. Peroxisome proliferator activated receptor ɣ, CCAAT/enhancer-binding protein α, and cell cycle status regulate the commitment to adipocyte differentiation. J. Biol. Chem., 272:21473-21478.
21. Steppan, C. M. et al. 2001. The hormone resistin links obesity to diabetes. Nature, 409:307-312.
22. Stevens, J. et al. 1981. Insulin resistance in adipocytes from fed and fasted obese rats: dissociation of two insulin actions. Mol. Cell. Biochem., 37:177-183.
23. Veerababu, G. et al. 2000. Overexpression of glutamine: fructose-6-phosphate amidotransferase in the liver of transgenic mice results in enhanced glycogen storage, hyperlipidemia, obesity, and impaired glucose tolerance. Diabetes, 49:2070-2078.
24. Vernon, R. G. et al. 2001. Signal of adiposity. Dom. Animal Endocrinol., 21:197-214.
25. Way, J. M. et al. 2001. Adipose tissue resistin expression is severely suppressed in obesity. J. Bio. Chem., 276:25651-25653.

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